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Aerobic degradation by a marine bacterium of pentachlorophenol

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AEROBIC DEGRADATION BY A MARINE BACTERIUM OF PENTACHLOROPHENOL

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ABSTRACT

AEROBIC DEGRADATION BY A MARINE BACTERIUM
OF PENTACHLOROPHENOL

by Colleen V. Hallen

Due to the widespread contamination by pentachlorophenol (PCP) in soil and groundwater, there is an urgent need to find microbes that can degrade PCP. In this study, 18 isolates obtained from the red alga Plocamium cartilageneum were investigated for their ability to degrade PCP. Only one isolate, 3b, was found to degrade PCP. Therefore, 3b appears to be the first PCP-degrading bacterium isolated from a marine habitat. This bacterium has not yet been taxonomically identified but is presently under study. PCP was utilized in this study because it is a toxic pollutant that poses a persistent environmental threat.

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OBJECTIVES OF THIS RESEARCH

Many kinds of halogenated organics are found in the ocean, particularly in species of the red alga Plocamium. Since these halogenated organics are naturally occurring, it has been speculated that microorganisms capable of degrading halogenated organics may be found associated with the alga. Microorganisms found in association with the alga might have evolved the ability to induce degradative pathways, through random mutation or various recombination modes, and to tolerate or even utilize these compounds. Consequently, if the microorganisms have the ability to degrade the naturally occurring halogenated compounds, they conceivably might also be able to degrade man-made halogenated compounds. The purpose of this research was then to determine whether microbes, in particular aerobic or facultative bacteria, isolated from Plocamium and exposed to naturally occurring halogenated organics are able to degrade man-made halogenated organics, such as PCP. Moreover, because most PCP degradation pathways that have been elucidated are reductive, this research endeavored to find an oxidative pathway. Another aim of the research was to ascertain whether a specific bacterial population on the alga was constant, with respect to time.

LITERATURE REVIEW

Halogenated Organics

It is widely believed that man has been the major contributor of halogenated compounds to the environment and that the environment was relatively free of organohalogens before man stepped in. This belief is completely inaccurate. More than 1500 different halogenated organic and, less commonly, inorganic chemicals are made and discharged into our biosphere by plants, marine organisms, insects, bacteria, and fungi through natural metabolic activity. In a few cases, the amounts of these naturally occurring halogenated compounds far exceed the amounts of the same chemicals from anthropogenic sources (7). Considering that organohalogens are found in so many different organisms from so many different habitats, it is clear that they have been around for thousands of years.

Although most of the naturally occurring halogenated organic compounds discovered so far contain chlorine or bromine, iodine and fluorine are also found in many different compounds. Iodine has been detected in organic compounds isolated from some marine algae, higher animals and humans while fluorine has been found in organic compounds isolated from bacteria and higher plants. For example, it has been determined that the oceans are a major

source of iodomethane, largely due to the vast quantities produced by giant kelp and marine algae. It was also reported that the antibiotic nucleocidin, which was isolated from a soil microbe in India, contains fluorine (7). Although iodine and fluorine compounds are not as prevalent in the environment as chlorine or bromine, they still make up a considerable and varied amount of the naturally produced halogenated organics.

While organohalogens are produced naturally in many different habitats, organisms in marine habitats are estimated to be the largest source of naturally produced organohalogenic compounds (7). Surprisingly, marine habitats are the least studied in terms of organohalogenated compounds. Up to 1987, 12,000 natural products had been isolated from terrestrial plants but only 500 from marine plants. With 500,000 species of marine animals, plants, and bacteria available, the marine habitat obviously has been an overlooked resource. An estimate of naturally occurring CH_3Cl from giant kelp alone is 2,000 tons a year. Since most marine organisms studied to date have been found to contain halogenated compounds, it is certain that the world's oceans hold vast numbers of yet undiscovered organohalogenated compounds (7).

Pentachlorophenol

Many chlorine compounds have found their way into the environment due to their extensive use by humans. Few man-made chemicals are as prevalent as those containing chlorine (5, 21). Organochlorine compounds are found in such common products as drugs, plastics and disinfectants. The most infamous compounds, including dichlorodiphenyl-trichloroethane (DDT), polychlorinated biphenyls (PCBs) and chlorofluorocarbons (CFCs), have already been prohibited or severely restricted in the United States. Chlorine is still used in making 96 percent of all pesticides (5). Many of these pesticides often end up polluting the groundwater and soil.

Pentachlorophenol (PCP) and its sodium salt (Na-PCP) are chlorine-containing compounds that were the second most widely used pesticides in the United States in the 1970s. They were registered by the US Environmental Protection Agency for use in controlling insects, bacteria, fungi, algae and as biocides in cooling water towers and in antifouling paints (13, 19). As a result of such widespread usage, PCP now contaminates bays, rivers and soils in and around the areas to which it was applied. PCP usage is now restricted to paper mills and oil recovery processes. Based on the Superfund Amendments and the Reauthorization Act of 1986 (SARA) which required a priority list of hazardous

substances, PCP became the thirty-first most hazardous substance (4).

The toxicity of pentachlorophenol is due to its action as a weak acid respiratory uncoupler. Weak acid uncouplers target biomembranes, particularly those of mitochondria, as nonsite-specific protonophoric uncouplers. Thus, they elicit their effects by abolishing the coupling of substrate oxidation to ATP synthesis (4, 20). In humans, prolonged exposure to PCP can result in adverse reproductive effects, contact dermatitis, persistent chloracne, as well as damage to the lungs, nervous system, liver, and kidneys. While skin penetration is the most dangerous route of exposure in humans, the most common exposure to PCP is inhalation in the work place (6). PCP is also acutely and chronically toxic to cold and warm water fish and strongly toxic to plants.

Bioremediation

Hazardous waste treatment methods fall into several general categories: physical, chemical, biological and thermal. There are problems associated with each of these methods. For example, physical methods can isolate the hazardous material from the non-hazardous, but additional treatment is frequently needed to destroy the hazardous material. Chemical methods often just modify the hazardous material, converting it chemically to even more hazardous by-products. While thermal methods are effective at

destroying the hazardous waste, this approach can be very expensive. High expenditures for thermal methods are often due to the need to transport the waste to the incinerator site and for the energy needed for incineration (23).

An alternative to these methods focuses on biological methods of hazardous waste treatment, usually defined as bioremediation. Bioremediation offers the possibility of detoxifying or degrading contaminants to harmless by-products (23). Bioremediation is also often the least expensive method of cleanup. Like other forms of hazardous waste processing, bioremediation is not a perfect treatment. If environmental conditions such as pH, temperature, water activity, redox potential and aeration are not adequate, microbial growth and survival will be adversely affected. Consequently, bioremediation may not occur at optimal rates (18).

Although there are many different requirements that need to be satisfied for successful bioremediation, it is still regarded as a very viable treatment method for certain situations. Microbes involved in bioremediation can be of one species, or two or more species can act as a consortium, in a stepwise process, to degrade a hazardous substance. In some cases, the hazardous substance may not be a good energy source for the microbe, therefore, a co-metabolite may be necessary for degradation. Suitable microbes are often

found at or near the contamination site. These indigenous organisms, exposed to hazardous substances for a long time, adapt and utilize the substances as a nutrient source.

Many different methods exist for accomplishing successful bioremediation in the field. These include land treatment (14), bioreactors (12), and *in-situ* treatment (8). Land treatment is a modified form of composting in which surface soils are manipulated by adding aeration, water, and nutrients in order to enhance degradation of the target substance. Land treatment is the cheapest of the three methods and is only used for lightly contaminated soils. Bioreactors provide a more controlled environment for the degradation process by removing the entire contaminated material and placing it in a tank or reactor. The reactor provides effective mixing of the contaminated water, sludge or soil. In addition, the nutrient and water addition, bacterial growth, temperature and pH are more readily controlled in this contained environment. Bioreactors can remediate highly contaminated soils in a relatively short amount of time but are also the most costly mode of bioremediation. *In-situ* treatment is a combination of the above treatments which remediates the contaminated material on-site, without having to excavate or remove it as is necessary for bioreactor treatment. *In-situ* treatment typically extracts contaminated ground water, adds nutrients

and oxygen, and injects it back into the contaminated soil. This enhances degradation by the constant circulation of the water. *In-situ* treatment is cheaper than bioreactor treatment and is effective for ground water and soil cleanup.

Degradation Mechanisms

There are several well known PCP degradation pathways, both reductive and oxidative (11, 16). A common reductive pathway for anaerobic bacteria and fungi is shown in Figure 1 (11). In the reductive pathway, the PCP loses chlorine atoms in a stepwise process. A common oxidative pathway is shown in Figure 2 (11). Oxygen reacts with the PCP, displacing the chlorine atom, and a hydroxyl group is formed.

Summary

Due to its high degree of toxicity, PCP is resistant to most microbial degradation. There have been a limited number of microbes found to degrade the halogenated organic. However, up to now only microbes isolated from soil (16, 25), freshwater, and sewage sludge have been described (3, 11). In this study a microbe isolated from a marine environment was used to degrade PCP. This is the only study known to this researcher to use a marine organism to degrade PCP.

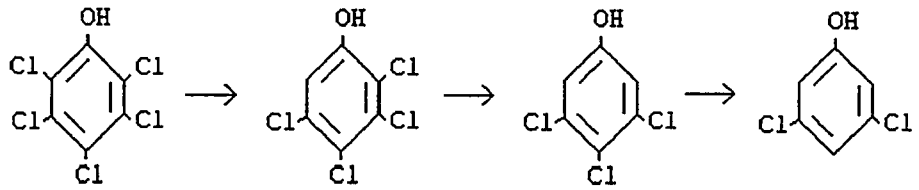

Figure 1. Reductive pathway.

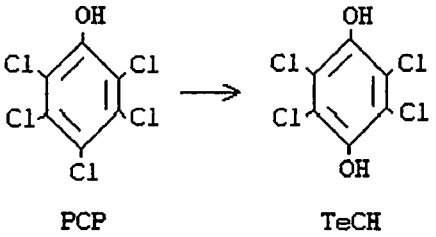

Figure 2. Oxidative pathway.

MATERIALS AND METHODS

Media and Halogenated Organic Chemical Preparation

The artificial seawater medium (ASW) contained (per liter of distilled water) 27.5 g, NaCl; 5.0 g, $MgCl_2$; 2.0 g, $MgSO_4$; 0.5 g, $CaCl_2$; 1.0 g, KCl; 1.0 g, $(NH_4)_2HPO_4$; and 1.0 mg, $FeSO_4$. The pH of the ASW medium was 6.8. Complete artificial seawater medium (CASW) was prepared by adding 1.0 g, yeast extract; 1.0 g, tryptose; and 0.5 g, glucose per liter of ASW. Complete artificial seawater agar contained 15.0 g of Bacto agar per liter of CASW.

O-F medium (Difco Manual) and Russell's Double Sugar medium (Difco Manual) were prepared with ASW instead of distilled water. The selective PCP medium (22) contained [(per liter of ASW) 0.065 g, K_2HPO_4; 0.017 g, KH_2PO_4; 0.100 g, $MgSO_4 \cdot 7H_2O$; 0.500 g, $NaNO_3$]; 50 ppm PCP, 1.5% purified agar and bromthymol blue (20 mg/L). The PCP was added after autoclaving. All media were sterilized by autoclaving at 121°C for 15 minutes.

Trichloroethylene (TCE), pentachlorophenol (PCP), or dibromodichloropropane (DBCP) were added to filter sterilized 0.1M NaOH to a concentration of 1000 ppm (1 mg/ml) and used as stock solutions. The stock solutions were refrigerated between uses. To obtain the desired

concentrations of the toxins in the reaction flasks, the stock solutions were added to sterile ASW or CASW, i.e., 1 ppm is equal to 0.02 ml of 1000 ppm PCP per 20 ml of ASW (0.02 ml/ 20 ml). Additional concentrations used were 10 ppm (0.2 ml/ 20 ml) 50 ppm (1.0 ml/19 ml) and 100 ppm (2.0 ml/ 18 ml). A fourth toxin, lindane, was prepared by adding crystals to 20 ml sterile ASW until they failed to dissolve (approximately 560 ppb).

Collection and Isolation of the Bacterial Strains

Initially specimens of Plocamium cartilageneum and Laurencia species were obtained in June, 1994. They were collected from a tide pool area at Davenport Landing, located 10 miles north of Santa Cruz, California. The time of the low tide, low tide measurement, temperature of the ocean proper and tide pool, and pH of the ocean proper and tide pool were recorded and are shown in Table 1. The algal specimens were taken from different places in the tide pools and from different parts of each alga. Only algae that were exposed to constant wave agitation were sampled. The algal samples were obtained with scissors, placed in a plastic bag with seawater, and put on ice for transport. All algal samples were refrigerated and used within 6-8 hours of being collected.

Sterile 50 ml Erlenmeyer flasks with plastic push-on caps were used as reaction flasks and filled with 20 or 18

Table 1. Measurements taken at algal collection sites.

Date of collection	Algae collected	Time of low tide	Low tide	Temp. of tide pools	Temp. of ocean	pH of tide pools	pH of ocean
6/24/94	both *	5:45 A.M.	-1.5	9.5-10°C	9°C	6-6.5	6.9
10/5/94	Plocamium	5:12 P.M.	-0.4	16°C	16°C	6.5	6
12/4/94	Plocamium	6:20 P.M.	-1.5	10°C	11°C	5.8	6
2/27/95	Plocamium	3:59 P.M.	-0.5	15°C	15°C	5.7	6

* Plocamium, Laurencia

ml of sterile ASW. TCE, PCP, or DBCP were used in 1 ppm, 10 ppm and 100 ppm concentrations. Lindane was also used. A small piece (about 3x5 mm) of Plocamium or Laurencia species was added to each flask. The final composition of the reaction flasks is summarized in Tables 2 and 3. These experiments were carried out in an incubator at 19 ± 2°C; the flasks were unshaken and kept in the dark to avoid photo-decomposition of the toxins.

After three days of incubation the flasks were visually inspected for signs of increased turbidity and a week after inoculation each flask was examined microscopically. Each reaction flask was then sampled for growth approximately every other day for two weeks, by spreading a 0.1 ml sample onto the surface of CASW agar plates. Isolated colonies of bacteria were taken from the spread plates and placed onto slants of CASW for further study.

Only the alga Plocamium was collected at Davenport Landing in October and in December of 1994 and in February of 1995 (See Table 1). Following the preliminary studies with the four halogenated compounds, it was decided that PCP would be used exclusively for the remainder of the study. The pH of the 1 ppm and 10 ppm flasks was approximately 7.0. Because there was such a large amount of PCP in the 100 ppm flasks, the pH was higher than 7.0 and, therefore, was adjusted with HCl to pH 7.0. The final composition of the

Table 2. Reagent composition of reaction flasks with Plocamium.

Flask #	ASW	CASW	Toxin	Toxin concentration
1		20 mls	none	
2		20 mls	none	
3		20 mls	TCE	1 ppm
4		20 mls	TCE	10 ppm
5		18 mls	TCE	100 ppm
6		20 mls	PCP	1 ppm
7		20 mls	PCP	10 ppm
8		18 mls	PCP	100 ppm
9		20 mls	DBPC	1 ppm
10		20 mls	DBPC	10 ppm
11		18 mls	DBPC	100 ppm
12		20 mls	lindane	560 ppb
45	20 mls		TCE	1 ppm
46	20 mls		TCE	1 ppm
47	20 mls		TCE	10 ppm
48	20 mls		TCE	10 ppm
49	18 mls		TCE	100 ppm
50	18 mls		TCE	100 ppm
51	20 mls		PCP	1 ppm
52	20 mls		PCP	1 ppm
53	20 mls		PCP	10 ppm
54	20 mls		PCP	10 ppm
55	18 mls		PCP	100 ppm
56	18 mls		PCP	100 ppm
57	20 mls		DBPC	1 ppm
58	20 mls		DBPC	1 ppm
59	20 mls		DBPC	10 ppm
60	20 mls		DBPC	10 ppm
61	18 mls		DBPC	100 ppm
62	18 mls		DBPC	100 ppm
63	20 mls		lindane	560 ppb
64	20 mls		lindane	560 ppb

TCE=trichloroethylene
PCP=pentachlorophenol
DBCP=dibromodichloropropane

Table 3. Reagent composition of reaction flasks with *Laurencia*.

Flask #	ASW	CASW	Toxin	Toxin concentration
13		20 mls	none	
14		20 mls	none	
15		20 mls	TCE	1 ppm
16		20 mls	TCE	10 ppm
17		18 mls	TCE	100 ppm
18		20 mls	PCP	1 ppm
19		20 mls	PCP	10 ppm
20		18 mls	PCP	100 ppm
21		20 mls	DBPC	1 ppm
22		20 mls	DBPC	10 ppm
23		18 mls	DBPC	100 ppm
24		20 mls	lindane	560 ppb
25	20 mls		TCE	1 ppm
26	20 mls		TCE	1 ppm
27	20 mls		TCE	10 ppm
28	20 mls		TCE	10 ppm
29	18 mls		TCE	100 ppm
30	18 mls		TCE	100 ppm
31	20 mls		PCP	1 ppm
32	20 mls		PCP	1 ppm
33	20 mls		PCP	10 ppm
34	20 mls		PCP	10 ppm
35	18 mls		PCP	100 ppm
36	18 mls		PCP	100 ppm
37	20 mls		DBPC	1 ppm
38	20 mls		DBPC	1 ppm
39	20 mls		DBPC	10 ppm
40	20 mls		DBPC	10 ppm
41	18 mls		DBPC	100 ppm
42	18 mls		DBPC	100 ppm
43	20 mls		lindane	560 ppb
44	20 mls		lindane	560 ppb

TCE=trichloroethylene
PCP=pentachlorophenol
DBCP=dibromodichloropropane

for all three collecting dates is summarized in Table 4. Incubation was at 19 ± 2°C and the flasks were unshaken and kept in the dark.

The flasks were sampled for growth as before at five and six days after collection and then every three to five days for approximately two weeks. Isolated colonies were transferred to slants of CASW and stored in the refrigerator.

All isolates were observed microscopically using a Leitz phase contrast microscope to determine cell morphology. The organisms were also tested for their Gram Stain reaction using the Hucker's modification of the Gram Stain procedure. The ability of the organisms to ferment glucose and/or lactose was tested on O-F medium and on Russell's Double Sugar medium. All isolates were then screened with the selective PCP medium. Based on these criteria, isolate 3b was selected for in depth PCP testing.

Experimental Design: PCP Degradation

A flow scheme of the experimental design for the degradation tests is shown by Figure 3. Bacterium 3b was grown on plates of sterile CASW agar for 24 hours at room temperature. The cells were harvested aseptically from the plate with a sterile loop and suspended in sterile ASW in 12 ml glass centrifuge tubes. Throughout the procedure the cells were kept on ice between steps. The cell suspension

Table 4. Reagent composition of reaction flasks.

Flask #	ASW	CASW	PCP concentration	Plocamium
1	20 mls		1 ppm	added
2	20 mls		1 ppm	added
3	20 mls		10 ppm	added
4	20 mls		10 ppm	added
5	18 mls		100 ppm	added
6	18 mls		100 ppm	added
7	20 mls		1 ppm	not added
8	20 mls		10 ppm	not added
9	18 mls		100 ppm	not added
10		20 mls	1 ppm	added
11		20 mls	10 ppm	added
12		18 mls	100 ppm	added
13		20 mls	none	added
14		20 mls	none	added
15		20 mls	1 ppm	not added
16		20 mls	10 ppm	not added
17		18 mls	100 ppm	not added
18		20 mls	none	not added
19		20 mls	none	not added

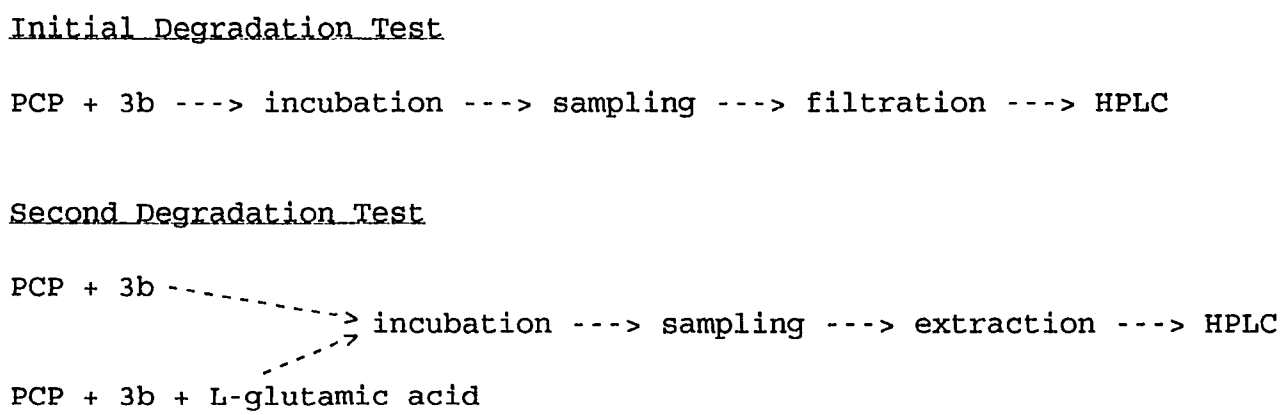

Figure 3. Flow schemes of experimental design for PCP degradation tests.

was centrifuged in a Model CL International Clinical Centrifuge (International Equipment Co.) at 1200 rpm for 10 minutes. The supernatant was removed and the pellet resuspended in sterile ASW. This washing process was repeated twice. The absorbance (600 nm) of the suspension was determined with a Spectronic 20 (Bausch and Lomb). At the same time, the bacteria were enumerated by the standard plate count using the spread-plate technique. The latter was performed using a 1 ml sample of the cell suspension with an absorbance of 0.7 at 600 nm. Colony counts were made after 48 hours of incubation at room temperature. A 1 ml sample of the suspension with an absorbance of 0.7 at 600 nm corresponded to 5.5×10^6 colony forming units per milliliter (cfu/ ml).

PCP solution was added to the ASW at 10 ppm and 50 ppm. Inoculation with 1 ml of the bacterial suspension, was analogous to 5.5×10^6 cfu/ 20 ml of medium. The final composition of the reaction flasks is summarized in Table 5. These experiments were carried out at 23 ± 2°C and the reaction flasks were shaken at 150 rpm in a Model G76 Gyratory Water Bath Shaker (New Brunswick Scientific). The reaction flasks were kept in the dark.

A 1 ml sample was removed from each reaction flask at the beginning (approximately 5 hours after bacterial addition), middle (53 hours) and end (100 hours) of the

Table 5. Reagent composition of reaction flasks in the initial degradation test using *Plocamium*.

Flask #	ASW	PCP	Bacterial suspension
1	19 mls	10 ppm	1 ml
2	19 mls	10 ppm	1 ml
3	18 mls	50 ppm	1 ml
4	18 mls	50 ppm	1 ml
5	20 mls	10 ppm	not added
6	20 mls	10 ppm	not added
7	19 mls	50 ppm	not added
8	19 mls	50 ppm	not added
9	19 mls	none	1 ml
10	19 mls	none	1 ml
11	20 mls	none	not added
12	20 mls	none	not added

degradation test for bacterial enumeration. Each sample was placed in a 12 ml glass centrifuge tube and centrifuged at 1200 rpm for 10 minutes. The supernatant was removed and the pellet washed twice. Throughout the procedure the cells were kept on ice between steps. The washed cells from each flask were enumerated by the standard plate count using the spread-plate technique. Colony counts were made after 2 days of incubation at room temperature.

High Pressure Liquid Chromatography (HPLC)

An additional 1 ml sample was removed from each flask twice a day for six days for HPLC testing. The first sample on day one was taken 30 minutes after inoculation with the bacterial suspension. There was approximately a seven hour interval between the first and second sample each day. The samples were added to a sterile 12 ml conical centrifuge tube and centrifuged at 1200 rpm for 10 minutes. The supernatant was removed and kept on ice. The supernatant was filtered with a Spartan-3 disposable syringe filter with a 0.45 mm pore size Teflon membrane (Sigma-Aldrich) attached to a sterile Luer-lok glass syringe. The filtrate was then placed in 2 ml amber glass vials with PTFE/silicone septa (Sigma-Aldrich). The vials were stored in a freezer at $0 \pm 2°C$.

For the internal standard, phenol was mixed with filtered, sterile ASW at 1 mg/ 2 ml, and 0.1 ml was added to

each vial. The phenol concentration in each vial was 50 ppm.

The vials of filtrate were removed from the 0°C storage one-half hour before injection. In order to prevent the precipitation of PCP into its sodium salt Na-PCP, 50 ml of HCl was added to each vial before injection. PCP was analyzed by HPLC with an HP Solvent Delivery System (Rainin). A Free-Flow Pulse Dampener (Alltech) was utilized to control pressure fluctuations. An Econosphere 5 m, 150 x 4.6 mm C18 chromatography column (Alltech) was used with an injection volume of 30 ml. The mobile phase was an isocratic mix of 75% methanol and 25% water. Detection was at 230 nm with a Dynamax Absorbance Detector Model UV-C (Rainin).

A subsequent degradation test was executed exactly like the first except for two components. The co-metabolite L-glutamic acid (Sigma) was used and extraction was utilized instead of filtration to prepare the samples for the HPLC. The final composition of the flasks is summarized in Table 6. A 1 ml sample from each flask was removed twice a day for 3 days then once a day for the remaining 3 days. The first sample on day one was taken approximately 60 minutes after inoculation of the bacterial suspension. There was approximately a 7 hour interval between the first and second samples taken on days with two samplings. Each 1 ml sample

Table 6. Reagent composition of reaction flasks in the second degradation test using Plocamium.

Flask #	ASW	PCP	L-glutamic acid	Bacterial suspension
1	19 mls	10 ppm	none	1 ml
2	19 mls	10 ppm	none	1 ml
3	18 mls	50 ppm	none	1 ml
4	18 mls	50 ppm	none	1 ml
5	20 mls	10 ppm	none	not added
6	20 mls	10 ppm	none	not added
7	19 mls	50 ppm	none	not added
8	19 mls	50 ppm	none	not added
9	19 mls	none	none	1 ml
10	19 mls	none	none	1 ml
11	20 mls	none	none	not added
12	20 mls	none	none	not added
13	19 mls	10 ppm	80 mg	1 ml
14	18 mls	50 ppm	80 mg	1 ml

was removed from the flask and placed in a sterile 15 ml glass tube (See Figure 4). The tubes were kept on ice between steps. The procedure was done in the dark. Two ml of pentane were added to each tube and mixed. The bottom layer (1 ml) was removed and placed in a second tube. Two ml of pentane were again added and mixed. The 4 ml of pentane (top layers) were combined. The pentane was then concentrated under a stream of compressed nitrogen gas, at room temperature. The resulting residue was dissolved in 1 ml of methanol/water (55%/45%) and placed in 2 ml amber glass vials with PTFE/silicone septa (Sigma-Aldrich). The vials were stored in a freezer at 0 ± 2°C. The internal standard, phenol, was mixed with methanol/water (55%/45%) instead of ASW.

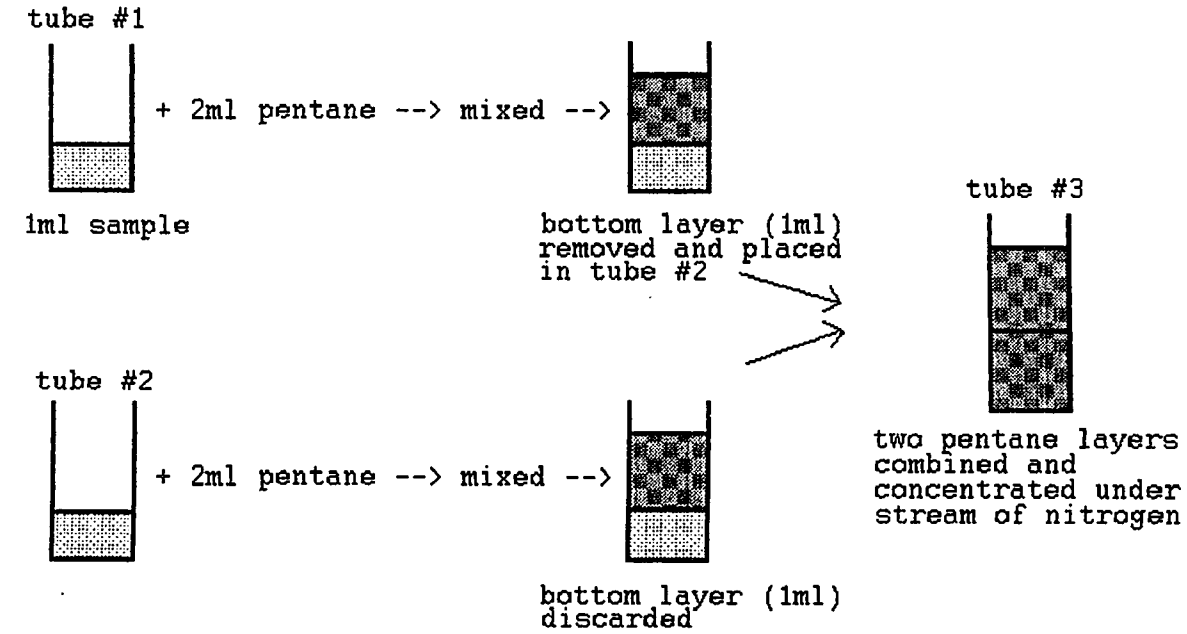

Figure 4. Extraction.

RESULTS

Table 7 shows the characteristics of the isolated bacteria in terms of their morphology, Gram stain reaction, glucose requirement, and PCP-degrading ability. All of the bacteria were Gram negative rods. The results of the Russell's Double Sugar and O-F tests indicated that none of the isolates fermented or respired glucose. Although the bacteria were collected at different times during a 12 month period, they showed little variation in these characteristics.

Of the 18 bacterial isolates, only one bacterium, 3b, tested positive on the selective PCP medium. 3b turned the medium entirely yellow and was chosen for PCP degradation.

The bacteria enumerated by spread-plate technique in the PCP degradation tests are shown in Tables 8 and 9. Table 8 describes the bacteria enumerated in the initial degradation test. In Table 8, flask #3 which contained 50 ppm PCP showed no growth of bacteria. Flask #4 (duplicate of flask #3) contained growth only on the last sampling day. Table 9 describes the bacteria enumerated in the second degradation test. In Table 9, flasks #3, 4 and 14, all of which contained 50 ppm PCP, showed no growth on the first sampling day. The flasks containing PCP at 50 ppm were the

Table 7. Characterization of bacteria isolated from Plocamium.

Bacterium	Collection date	Morphology	Gram Stain	Glucose respiration/ fermentation	PCP-degrader
46	6/24/94	short rods	negative	no	no
48	6/24/94	rods	negative	no	no
49	6/24/94	very short rods	negative	no	no
52	6/24/94	rods	negative	no	no
56	6/24/94	very short rods	negative	no	no
56p	6/24/94	curved rods	negative	no	no
59	6/24/94	short rods	negative	no	no
60	6/24/94	rods	negative	no	no
62	6/24/94	very short rods	negative	no	no
64	6/24/94	very short rods	negative	no	no
3b	10/5/94	rods	negative	no	yes
4a	10/5/94	short rods	negative	no	no
6a	10/5/94	short rods	negative	no	no
3aD	12/4/94	short rods	negative	no	no
4aD	12/4/94	short rods	negative	no	no
2aF	2/27/95	short rods	negative	no	no
2bF	2/27/95	short rods	negative	no	no
3aF	2/27/95	short rods	negative	no	no

Table 8. Bacteria enumerated by spread-plate technique in initial degradation test.

Flask #	PCP (ppm)	Dates of Sample Removal 5/18	5/20	5/23
1	10	2.7×10^5 cfu	4.9×10^6 cfu	1.6×10^6 cfu
2	10	3.6×10^5 cfu	7.2×10^6 cfu	1.5×10^6 cfu
3	50	none	none	none
4	50	none	none	8.5×10^5 cfu
9	none	4.0×10^5 cfu	1.8×10^5 cfu	1.2×10^6 cfu
10	none	3.8×10^5 cfu	2.5×10^5 cfu	2.6×10^6 cfu

#1 and #2 are duplicates
#3 and #4 are duplicates
#9 and #10 are duplicates

Table 9. Bacteria enumerated by spread-plate technique in second degradation test.

Flask #	PCP (ppm)	Dates of Sample Removal 8/28	8/30	9/2
1	10	1.2×10^6 cfu	5.4×10^7 cfu	1.5×10^7 cfu
2	10	5.0×10^6 cfu	3.3×10^7 cfu	2.6×10^7 cfu
3	50	none	2.8×10^7 cfu	2.1×10^7 cfu
4	50	none	1.7×10^7 cfu	1.3×10^7 cfu
9	none	1.4×10^7 cfu	2.2×10^7 cfu	1.8×10^7 cfu
10	none	6.3×10^6 cfu	1.7×10^7 cfu	1.6×10^7 cfu
13	10	3.5×10^6 cfu	1.3×10^7 cfu	4.8×10^7 cfu
14	50	none	3.1×10^7 cfu	5.7×10^7 cfu

#1 and #2 are duplicates
#3 and #4 are duplicates
#9 and #10 are duplicates
#13 and #14 are duplicates

only flasks that did not exhibit bacterial growth for one or all of the sampling dates.

The bacteria enumerated in the initial degradation test in Table 8 generally increased by a factor of 10 by the second sampling day. By the third sampling day, though, the quantity of bacteria seemed to remain constant. This increase followed by a leveling off was also found to be true for the subsequent degradation test as shown by Table 9.

The results of the HPLC analysis of PCP in the initial PCP degradation test are summarized in Figures 5 and 6. Figure 5 describes the degradation of 50 ppm of PCP. In Figure 5, one hour after bacterial addition, Degradation Bars 1 and 2 were close in area to Control Bar 1. All bars showed a large amount of detectable PCP. After eight hours, Degradation Bar 2 was equal in area to Control Bars 1 and 2. At this point there was still a large amount of detectable PCP. There was a significant change in the amount of PCP detected between 8 and 24 hours. The amount of PCP decreased dramatically in both the degradation and control bars. By 94 hours there was little PCP detected.

Figure 6 describes the degradation of 10 ppm of PCP. In Figure 6, one hour after bacterial addition, Degradation Bar 1 was close in area to Control Bars 1 and 2. All the bars showed a large amount of detectable PCP. After eight

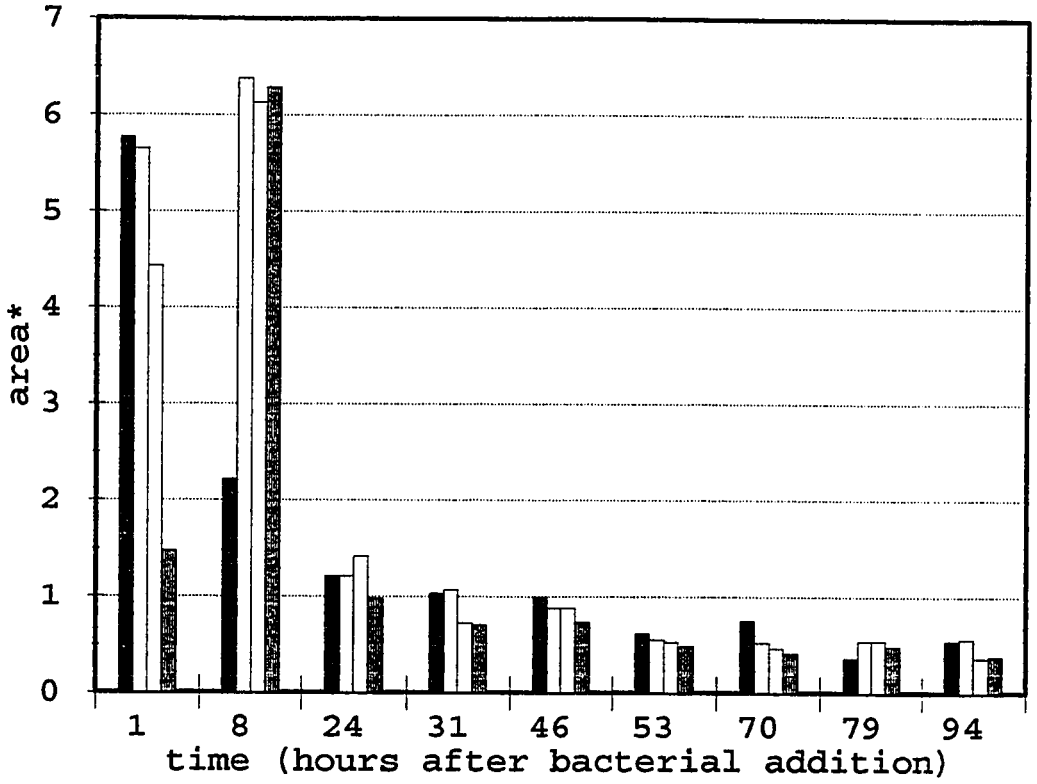

Figure 5. Degradation of 50 ppm PCP.

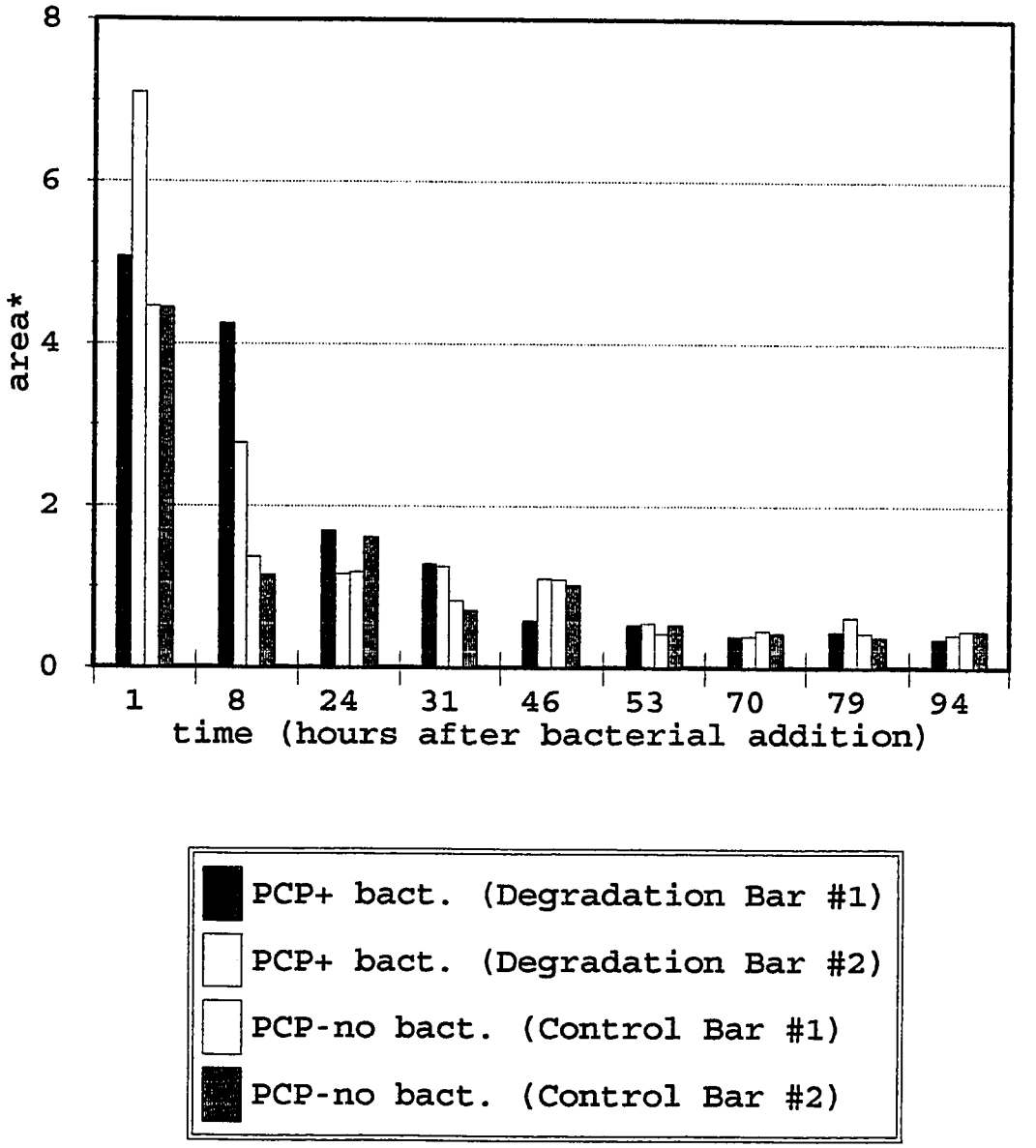

Figure 6. Degradation of 10 ppm PCP.

hours, only Degradation Bar 1 still showed a large amount of detectable PCP. Degradation Bar 2 had sharply decreased as had both control bars. At 24 hours, the degradation and control bars both displayed low PCP levels. By 94 hours there was hardly any PCP detected.

Figure 5 illustrates the testing of 50 ppm of PCP for possible degradation, and Figure 6 illustrates the testing of 10 ppm of PCP for possible degradation. In Figure 6, there was a decrease in the amount of PCP detected in all bars after only one hour while in Figure 5, there was a decrease in the amount of PCP detected in all bars after eight hours. Figures 5 and 6 show decreases in PCP in both the degradation bars and the control bars.

The results of the HPLC analysis of PCP in the second PCP degradation test are summarized in Figures 7-10. Figure 7 describes the degradation of 50 ppm of PCP without L-glutamic acid. In one hour after bacterial addition, Degradation Bar 2 was approximately equal in area to Control Bars 1 and 2. All the bars showed a large amount of detectable PCP. Throughout the remainder of the run, the degradation bars and the control bars were somewhat equal to each other. At 65 hours, though, Control Bar 2 did show a sharp decrease in the amount of PCP detected, but by 91 hours the bar had recovered.

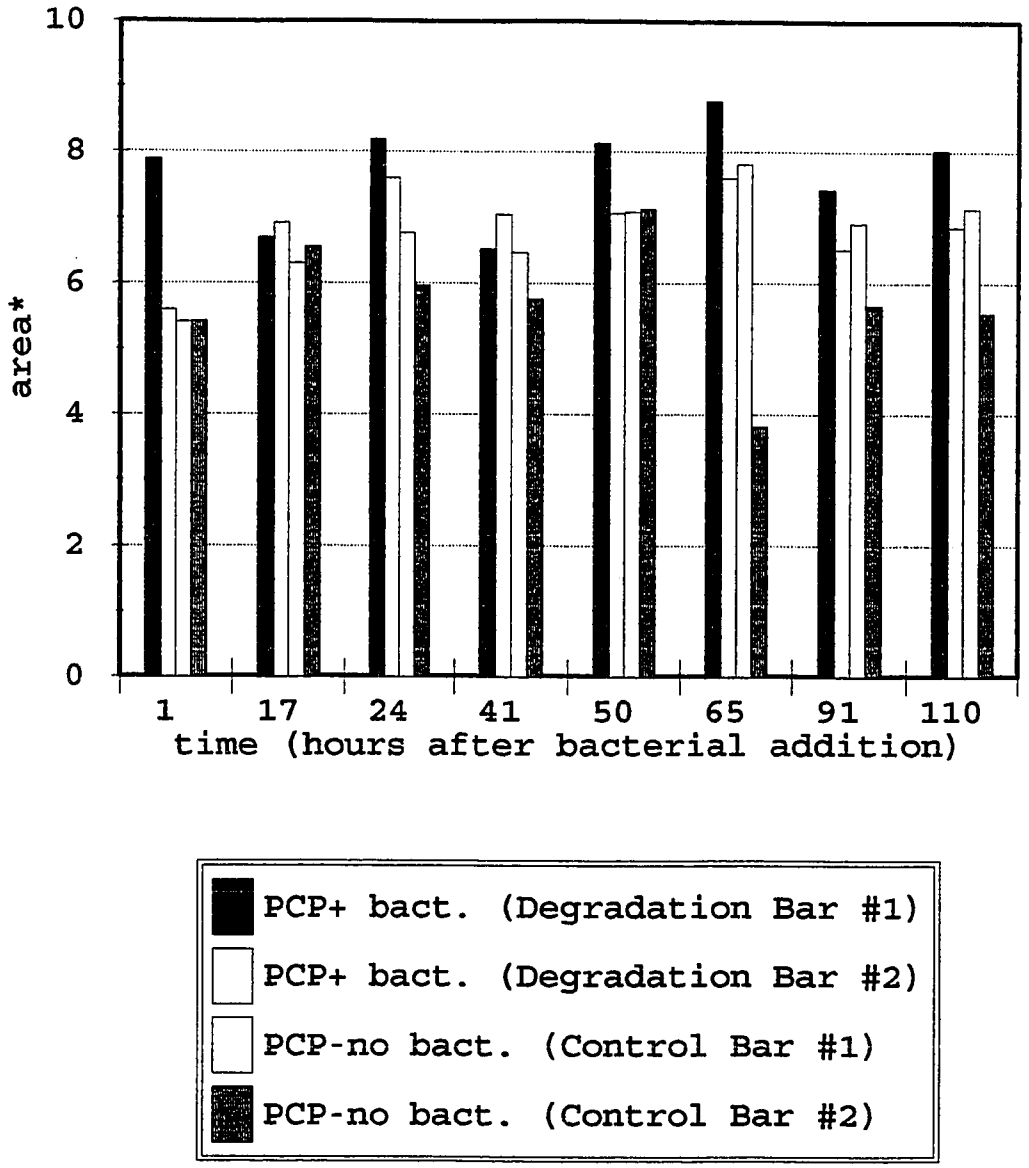

Figure 7. Degradation of 50 ppm PCP without L-glutamic acid.

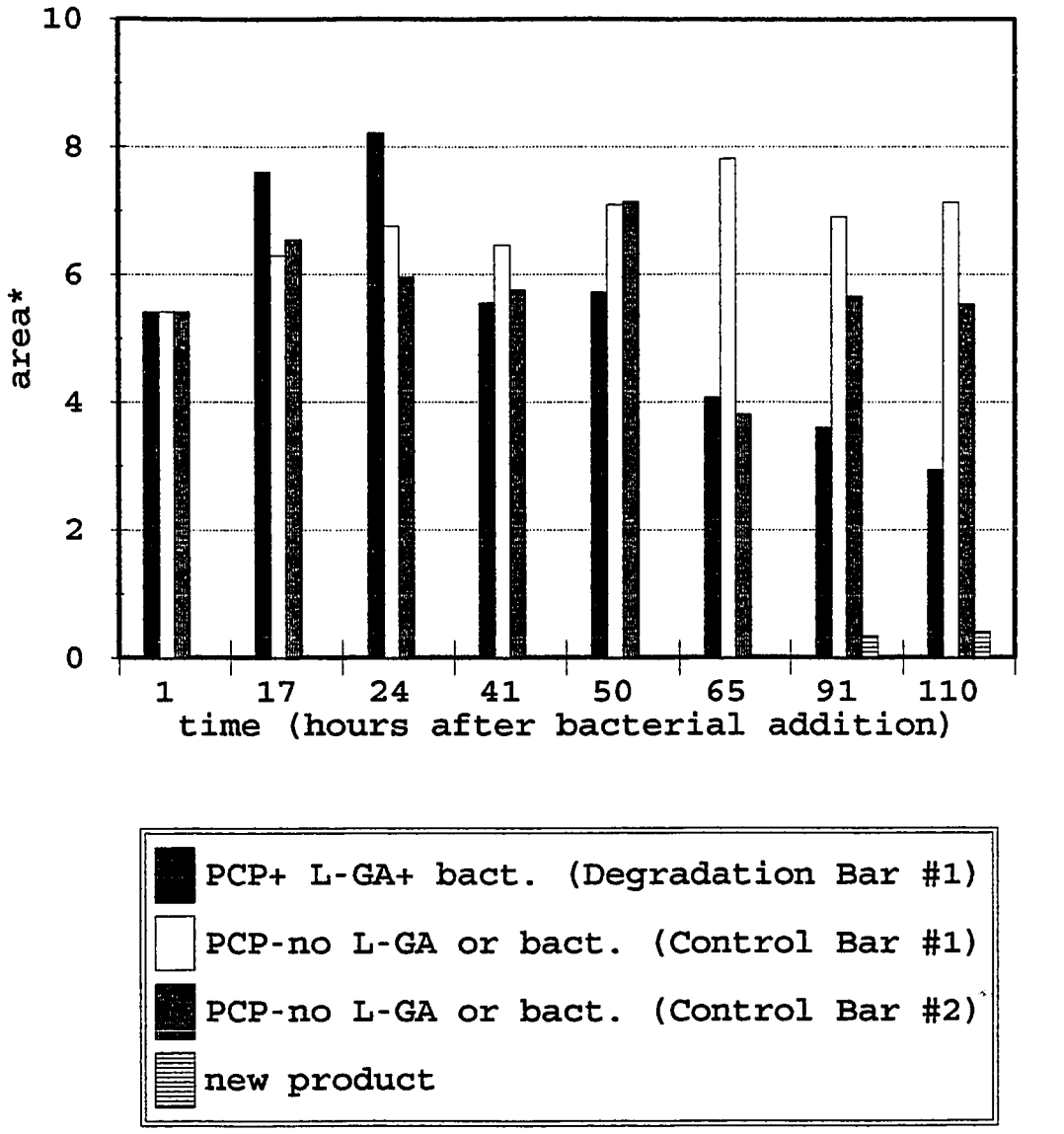

Figure 8. Degradation of 50 ppm PCP with L-glutamic acid.

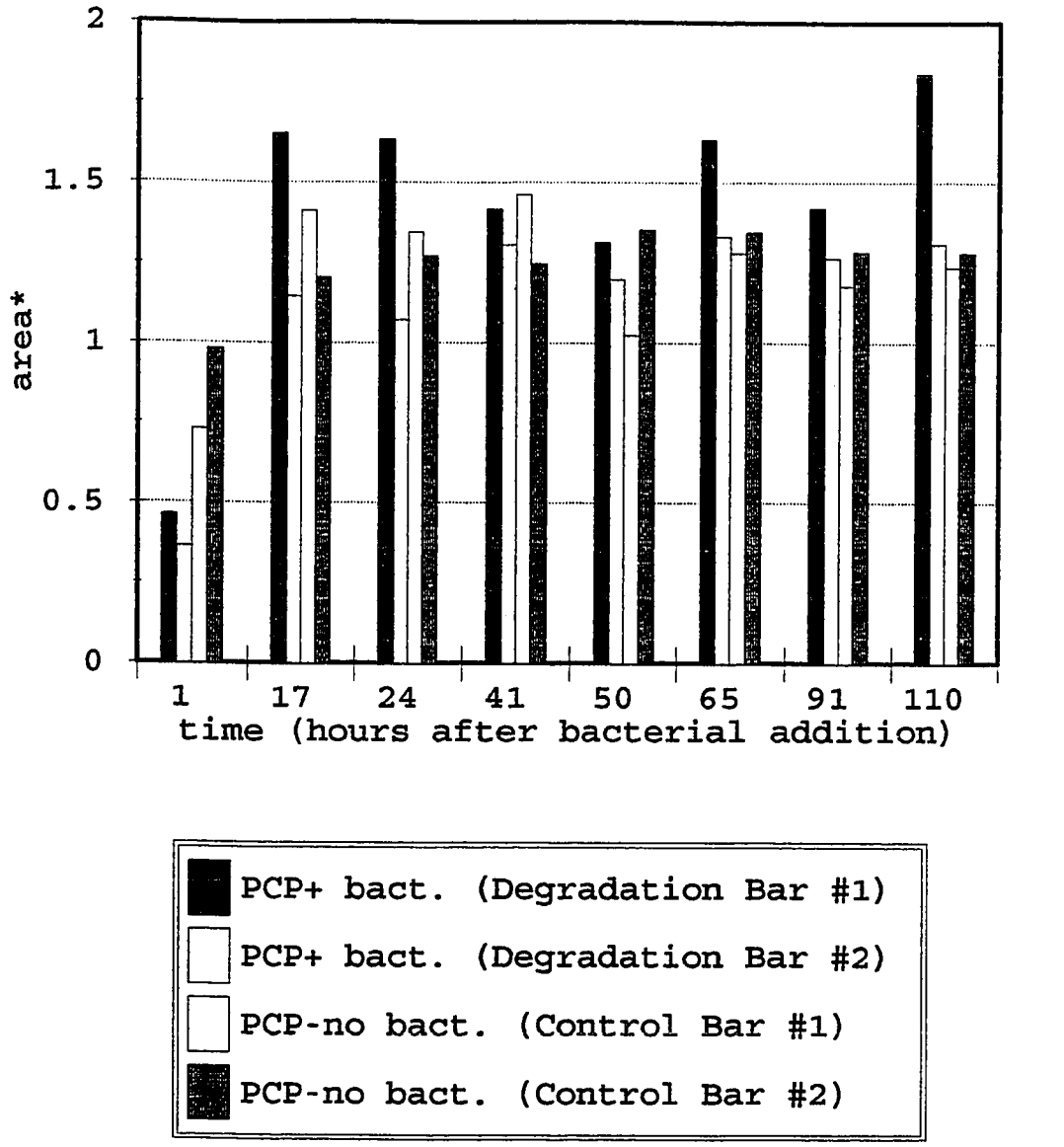

Figure 9. Degradation of 10 ppm PCP without L-glutamic acid.

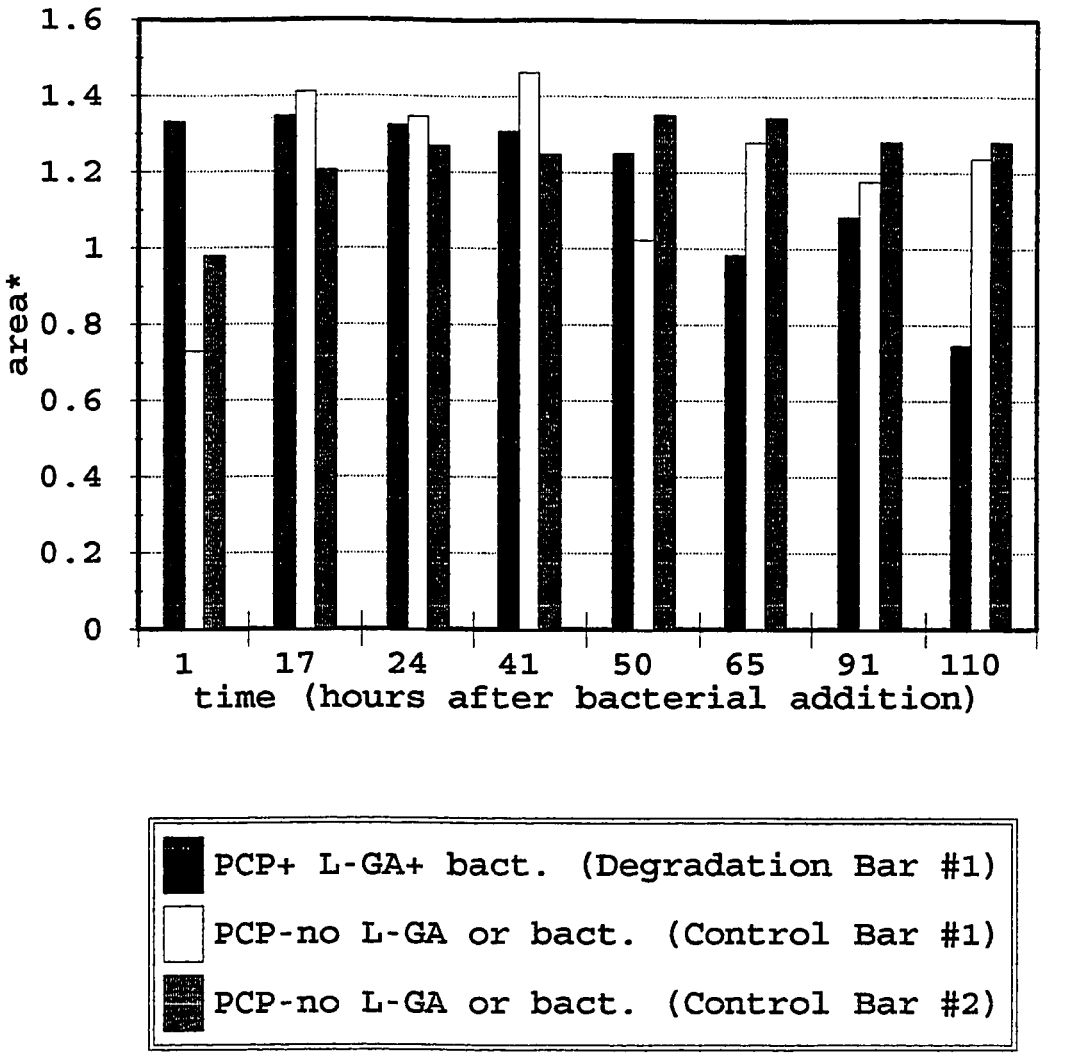

Figure 10. Degradation of 10 ppm PCP with L-glutamic acid.

Figure 8 describes the degradation of 50 ppm of PCP with L-glutamic acid. Here, one hour after bacterial addition, Degradation Bar 1 was equal in area to Control Bars 1 and 2. All the bars showed a large amount of detectable PCP. From 1 to 24 hours, all the bars remained somewhat equal to each other and did not change much in amount of detectable PCP. Between 24 and 50 hours Degradation Bar 1 decreased slightly; the control bars remained the same. Within 50 to 110 hours there was a enormous change in the amount of PCP detected in the degradation bar. The degradation bar decreased by more than half its original area while the control bars remained the same. Between 65 and 91 hours a new bar appeared, which got slightly larger by 110 hours. At 65 hours, though, Control Bar 2 did show a sharp decrease in the amount of PCP detected, but by 91 hours the bar had recovered.

Figures 7 and 8 both exhibit the testing of 50 ppm of PCP for possible degradation. The degradation in the figures differed in that in Figure 8 a co-metabolite, L-glutamic acid, was used. While Figure 7 showed no decrease in either the degradation or control bars, Figure 8 showed a major decrease in the degradation bar.

Figure 9 describes the degradation of 10 ppm of PCP without L-glutamic acid. In Figure 9, one hour after bacterial addition, Degradation Bars 1 and 2 had smaller

areas than Control Bars 1 and 2. All the bars showed a relatively small amount of detectable PCP. By 17 hours, though, all the bars had increased and showed a large amount of detectable PCP. Throughout the rest of the run, the degradation bars and the control bars remained somewhat equal to each other and all showed a large amount of detectable PCP.

Figure 10 describes the degradation of 10 ppm of PCP with L-glutamic acid. In Figure 10, Degradation Bar 1 had a slightly larger area than Control Bars 1 and 2. However, by 17 hours, the control bars had increased and exhibited a large amount of detectable PCP. All the bars showed a large amount of detectable PCP. From 1 to 50 hours, all the bars remained somewhat equal to each other and did not change much in the amount of PCP detected. Between 50 and 110 hours there was a change in the amount of PCP detected in the degradation bar. The degradation bar decreased by almost half its original area while the control bars remained the same.

Figures 9 and 10 both show the testing of 10 ppm of PCP for possible degradation. As was the case in Figure 8, in Figure 10, a co-metabolite, L-glutamic acid, was used. While Figure 9 showed no decrease in either the degradation or control bars, Figure 10 showed a major decrease in the degradation bar.

Figure 8 and Figure 10 both demonstrate the testing of PCP with the co-metabolite, L-glutamic acid, for possible degradation. The 50 ppm PCP degradation bar in Figure 8 showed a greater decrease in the amount of PCP than the 10 ppm PCP degradation bar in Figure 10. The 50 ppm PCP bar in Figure 8 also showed a new bar appearing, while Figure 10 did not show a new bar.

The initial PCP degradation test utilized filtration to prepare the samples for HPLC, and as shown in Figures 5 and 6 the degradation bars and the control bars decreased. The second PCP degradation test used extraction to prepare the samples for HPLC, and as indicated by Figures 7-10 only the degradation bars decreased.

DISCUSSION

Pollution of the environment is one of the biggest problems facing the world. Mankind cannot produce harmful products and expect them to disappear. Identifying microorganisms with the ability to control and/or eliminate man-made pollutants is a high priority in research today. This research addressed the problem of pollution by the man-made chlorinated organic, PCP. This researcher used a bacterium, found on the alga Plocamium, to degrade PCP. These results show that there is great potential in finding microorganisms that are able to degrade man-made pollutants.

The bacterium, 3b, was chosen for the PCP degradation tests based on the results of the selective PCP medium. The selective PCP medium contains both PCP and bromthymol blue, which is a pH indicator. The test is based on the use of PCP as the sole carbon source. This test uses washed cells, in order to avoid adding anything extra that could cause a change in pH or that could be used as a nutrient in lieu of the PCP. The selective medium is initially blue. If an organism is added that can metabolize PCP, it might cleave a chlorine off the PCP. If this metabolism produces HCl, the medium will become acidic and turn from blue to yellow. Isolate 3b turned the whole plate yellow. 3b did not have an affect on the control plate (no PCP). Since only washed

cells were used in the test, it is unlikely that an organism other than 3b caused the pH change. Also, the most probable initial step in the pathway of aerobic and anaerobic PCP degradation is cleavage of a chlorine (1, 15).

The alga Plocamium was sampled at different intervals over a 12 month period. This was done in order to determine whether the bacterial population found on the alga was constant or changing. The isolated bacteria were found to have very similar characteristics, in terms of shape, Gram stain, and glucose use. Therefore, it is evident that the bacterial population was fairly constant. This would suggest that certain bacteria found on the alga may have evolved the ability to tolerate or even to utilize the halogenated organics produced by the alga.

Samples are prepared for HPLC analysis by two major methods: filtration and extraction. This research utilized both methods. Filtration was the method used in the initial degradation test. Figures 5 and 6 demonstrate that the PCP decreases immediately in both the flasks with bacteria and in the control flasks. The initial samplings showed some PCP, but the succeeding samples showed very low amounts of PCP. It is possible that after the initial sampling (about an hour after addition of the bacteria) the PCP might have absorbed to the bacteria and been filtered out. Due to the inconclusive results of the filtration method, extraction

was used for the second degradation test. As demonstrated by Figures 7-10, the extraction method was effective because it did not remove the PCP from the sample.

The HPLC sample preparation method utilized with the second degradation test was extraction using pentane as the extractant. When compared to the filtration method used for the initial degradation test, extraction achieved the most reasonable and consistent results. The test with filtration showed a reduction of PCP in all the flasks with bacteria and without bacteria (Figures 1 and 2). The test with extraction showed steady PCP amounts in the flasks without bacteria. A study of PCP degradation by Briglia *et al.* (2) employed extraction with acetone-HCl as the extractant followed by gas-liquid chromatography. The study tested the PCP-mineralizing ability of Rhodococcus chlorophenolicus in two types of soils. The results showed high PCP mineralization in both soils. The use of the gas chromatograph with hexane as the extractant was used by Lamar and Dietrich (9). The study utilized lignin-degrading fungi in the breakdown of PCP-treated wood. All four fungi caused notable wood weight losses and decreases in PCP amount. Premalatha and Rajakumar (17) tested PCP degradation using the extraction method with HPLC and an ethyl acetate extractant. Five Pseudomonas species were analyzed for PCP-degrading ability. Pseudomonas aeruginosa

exhibited very high PCP degradation with glucose as a co-substrate.

The first test of PCP degradation by 3b did not involve the use of a co-metabolite. The second test using 3b employed L-glutamic acid as a co-metabolite (10). In the second test, the two flasks that contained both PCP and L-glutamic acid were the only flasks that showed degradation. PCP was degraded in the presence of the co-metabolite at both PCP concentrations and was not degraded when the co-metabolite was not present.

The co-metabolite L-glutamic acid was needed in this research in order to achieve successful PCP degradation. PCP degradation did not occur in flasks which lacked L-glutamic acid. Many other studies also found that certain co-metabolites were necessary to attain PCP degradation. Topp *et al.* (24) tested the abilities of glucose and glutamate to stimulate PCP degradation. It was found that adding either glucose or glutamate produced better PCP degradation. The study by Premalatha and Rajakumar (17) determined that carbon sources such as peptone, malt extract, yeast extract, and nutrient broth supported the growth of the organism but did not support PCP degradation. Glucose was the only carbon source that served as a suitable co-substrate. There were 4 different substrates tested for use as a co-metabolite with PCP in the experiment by Ryding

et al. (21). The co-metabolites were methane, phenol, glucose and toluene. It was shown that the toluene-enriched mixture displayed the best PCP-degradation.

Radehaus and Schmidt (19) isolated a novel Pseudomonas that could degrade up to 160 mg/L of PCP. The study found that higher concentrations of PCP adversely affected the growth rate of the organism. It was demonstrated by Wall and Stratton (25) that complete degradation of PCP occurred from an initial concentration of 10 mg/L to non-detectable levels. This research showed that 3b could degrade 10 ppm (10 mg/L) and 50 ppm (50 mg/L) of PCP. This research successfully used low to intermediate PCP concentrations that were known, in these and other studies, to be degradable by bacteria.

Studies by Middaugh *et al.* (13), Premalatha and Rajakumar (17) and Resnick and Chapman (20) found organisms capable of degrading PCP from PCP-contaminated soil. An organism originally isolated from freshwater sediment containing PCP was shown to degrade PCP in an experiment by Brown (3). All the above organisms were isolated from soil and freshwater samples. This research used an organism isolated from a marine sample.

A study by Middaugh *et al.* (13) found that degradation from a measured concentration of 39-40 mg/L of PCP to 0.0006 mg/L of PCP was observed within 5 days. A degradation

mixture containing 100 mg/L of PCP was found by Resnick and Chapman (20) to be completely degraded in 5 hours. This research displayed a much slower rate of degradation of PCP. The amount of PCP decreased by almost half at 10 mg/L PCP and more than half at 50 mg/L PCP within 6 days.

A preliminary PCP degradation test was first attempted using bacterial isolates from the collection in June. No detection method, such as HPLC, was used in this test. It was thought that the increased growth of the bacteria in the flasks might provide enough of a visual indication of PCP degradation. The test was terminated when it was determined that growth of the bacteria was not dense enough to be a good visual indicator of PCP degradation. Moreover, observation of turbidity did not confirm degradation, since disappearance of PCP and appearance of breakdown products was not possible. The HPLC was used for the subsequent tests.

The bacteria enumerated by spread-plate technique in the two degradation tests did not show significant increase in number. Even in the second test, in the flasks with L-glutamic acid, the bacteria did not notably increase. In the pre-HPLC degradation test described above, the growth of the bacteria was not dense enough to be a good visual indicator of PCP degradation. The bacteria may be using PCP as a carbon source as well as be suppressed by the toxin

and/or possibly the degradation end product(s). This may account for the small increase in number of bacteria shown in the two degradation tests using HPLC. The future degradation tests using PCP and L-glutamic acid need to extend the duration of the test to observe whether or not the bacteria increases more significantly in number and/or increase the PCP concentration.

Spartan-3 disposable syringe filters with a 0.2 mm pore size Teflon membrane were first used to filter bacteria from the supernatant. The pore size was found to be impractical for such a use because they clogged after filtering only about 0.1 ml of the 0.5 ml sample. Alternatively, syringe filters with a 0.45 mm pore size proved satisfactory. It was necessary to filter about 0.1 ml of methanol through each filter before filtering the supernatant because the filter seemed to be moderately hydrophobic. Filtering the methanol first allowed the water-based supernatant to then be filtered. Teflon filters were needed because halogenated compounds tend to stick to filters having other chemical compositions.

An HPLC chromatography column by Waters was to be used for this experiment. Calibration curves for this column were run and the peaks for PCP and phenol were separate and very distinct. The first degradation test was performed and the first few samples were run on this column. The peaks

for PCP and phenol were no longer separate and distinct but were close together and overlapping. This problem did not rectify itself, and it was determined that it was caused by channeling in the column due to the absence of a pulse dampener.

Conclusion

In this investigation, a bacterium isolated from a marine habitat was shown aerobically to degrade up to 50 ppm of PCP in the presence of L-glutamic acid. Although PCP has already been found to be degraded by organisms exposed to man-made sources of PCP, this is the first study known to have isolated a PCP-degrading organism that was only exposed to naturally occurring sources of halogenated organic compounds. This organism had not been previously exposed to PCP. Furthermore, unlike all other studies known to this investigator, this research demonstrated that an organism isolated from a marine environment could degrade PCP.

Future Work

In terms of future research with 3b, there are many exciting areas to explore. For example, it will be interesting to speciate 3b to ascertain whether it is a known species. The bacterium must also be analyzed to determine its ability to degrade other halogenated organics. Many organisms have been found that can degrade more than one halogenated organic. There are also other co-

metabolites that have been used to obtain successful PCP degradation. These co-metabolites should be tested with 3b.

The new product resulting from the co-metabolism of PCP needs to be analyzed, and once identified, compared to products in known PCP degradation pathways. Further studies must also vary growth conditions such as temperature, oxygen relationship as well as the concentrations of the PCP and L-glutamic acid in order to obtain the most efficient route for PCP degradation by 3b.

Marine habitats need to be increasingly utilized as sources of organohalogen-degrading microorganisms. Marine sponges, corals, mollusks, jelly fish, as well as algae (7), have all been found to contain organohalogens. The marine realm should not be overlooked.

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